

THE CONSTRUCTION AND CHARACTERIZATION OF VACCINES EXPRESSING
THE PRE-MEMBRANE AND GLYCOPROTEIN E ANTIGENS OF THE
WEST NILE VIRUS

By
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DEDICATION:

I would like to dedicate this Capstone Project to my loving grandparents, Jack and Susan Jacobs. Without their care and influence, I would not be who I am today, and might not have had the opportunity to construct this undergraduate thesis for the Sally McDonnell Barksdale Honors College at The University of Mississippi.

ABSTRACT

RYAN TYLER JOHNSON: The Creation and Characterization of Vaccines Expressing the Premembrane and Glycoprotein E Antigens of the West Nile Virus
(Under the direction of Dr. Wayne Gray)

The West Nile Virus (WNV) is a positive polarity, single-stranded RNA virus belonging to the family *Flaviviridae*. In this family, there are several globally relevant human pathogens including dengue fever virus (DENV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), the newly emerging Zika virus, and the West Nile Virus (WNV). WNV, like other Flaviviruses, is spread by an arthropod vector, and can cause West Nile fever and West Nile encephalitis and meningitis, and is now considered to be endemic in Africa, Asia, Europe, the Middle East, and the United States. No vaccine is currently available to prevent WNV infection, and it is clear that one is needed.

This thesis aims to provide significant, relevant background information, pathophysiology, epidemiology, and highlight various vaccination strategies that have been attempted or are currently being researched to combat WNV. Furthermore, this paper aims to provide a plausible solution to WNV with two different vaccines that were created: a DNA and recombinant Varicella Zoster Virus (rVZV) vaccine, both expressing the pre-membrane and glycoprotein E antigens of the virus. The DNA vaccine achieved successful transfection and expression in Vero cells.

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LIST OF SYMBOLS/ABBREVIATIONS

WNV	West Nile Virus
PrM	Premembrane
gE	glycoprotein E
VZV	Varicella Zoster Virus
rVZV	Recombinant Varicella Zoster Virus
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
HIV	Human Immunodeficiency Virus
SIV	Simian Immunodeficiency Virus
ELISA	Enzyme-linked immunosorbent assay
PCR	Polymerase chain reaction
RT-PCR	Reverse-transcriptase polymerase chain reaction
IV	Intravenous
CNS	Central Nervous System
BBB	Blood Brain Barrier
MHC	Major Histocompatibility Complex
JM109	Bacterial Strain of E.coli
Amp.	Ampicillin (antibiotic)
IPA	Isopropanol
TE	“Tris-EDTA”, used to store DNA & RNA
EtBr	Ethidium Bromide

BamH1	“Bacillus amyloliquefaciens” – restriction enzyme
D2H2O	Deionized H2O (water)
rSAP	Shrimp Alkaline Phosphatase
Kan.	Kanamycin (antibiotic)
EB	Elusion Buffer
BSA	Bovine Serum Albumin
PBS	Phosphate Buffered Saline
RPM	Rotations per Minute

INTRODUCTION

In this study, the area of focus is the creation of two possible modes of vaccination for the West Nile Virus (WNV), which plagues many parts of the world and is now endemic in both hemispheres. The WNV is a positive, single-stranded RNA Flavivirus. Like other flaviviruses, the WNV is spread by means of an arthropod vector, with birds being the reservoir hosts, humans being the dead-end host, and mosquitos being the vector that ensures the cycle of infection.

The types of vaccines that were created and that will be discussed in this paper are a DNA vaccine and a recombinant Varicella Zoster Virus (rVZV) vaccine. Both vaccine types will be expressing the Pre-membrane (prM) and the glycoprotein E (gE) antigens of the WNV. Both antigens are structural proteins that are vital to the virus's formation and successful infection into the host cell. The DNA vaccine is a relatively new vaccine construct that has been proven to be safe, reliable, and cost-effective. It involves inserting the antigens of a disease into a vector, which gives protection to the individual without the injection of infectious agents like traditional vaccine methods. The rVZV vaccine is a relatively new construct that some researchers are starting to work with in the field of virology. The VZV vaccine has seen large scale success since its inception and has been proven to be safe and effective. The vaccine genome is large enough to insert genes of interest and still be effectively transfected. The aim of this paper is to create and characterize these vaccine types in the hope that one day a safe and effective vaccine for the WNV will be created, approved, and used on a large scale.

CHAPTER I: WNV BACKGROUND INFORMATION, CLINICAL USE, PATHOPHYSIOLOGY, EPIDEMIOLOGY, AND VACCINATION STRATEGIES

INTRODUCTION

West Nile Virus (WNV) is an RNA virus that is a member of the Japanese encephalitis antigenic complex of the family *Flaviviridae*. It is commonly found in Africa, Europe, the Middle East, North America, and West Asia. (1) Like other flaviviruses, it is spread by means of an arthropod vector. In nature, it is maintained in a cycle involving transmission between mosquitoes and birds, with birds being the reservoir hosts and mosquitoes being the vector of infection. Humans are referred to as the “dead-end hosts”, because while they can become infected, they cannot transmit the virus any further. WNV was first isolated in a woman in the West Nile district of Uganda in 1937, earning it its name. In 1999, it was reported in New York which produced a large outbreak that spread throughout the United States in the following years. Outbreak sites are observed to occur on major bird migratory routes.

MOLECULAR PROPERTIES OF WNV:

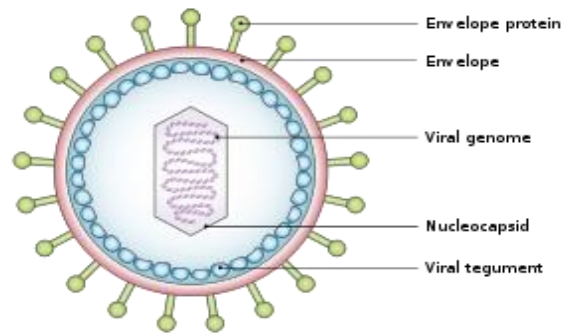


Figure 1 - WNV Structure

The structure of the West Nile virus is vital knowledge to understand how the virus operates and conveys infection in an individual (*Figure 1*). The outer layer of the virus is composed of a lipid bilayer and serves as the envelope of the virus. The presence of an envelope tells us that the virus is unstable in the external environment, which is why it requires a vector to successfully transmit to a host. The spikey protrusions seen on the surface of the virus are the glycoproteins that project out from the envelope, which helps the virus bind to the host cells. Tucked away safely in the center of the virus is the nucleocapsid, which is composed of two main parts: the capsid, which is the icosahedral-shaped protein shell that protects the genetic information on the inside, and the RNA, the single-stranded genetic information of the virus. (21)

The WNV proteins of interest in this study are the Pre-membrane and glycoprotein E (PrMgE) antigens. The pre-membrane protein of flaviviruses is a structural protein, and it plays an important role of conformational folding of the envelope protein of the virus and helps protect it against pre-mature fusion in the acidic vesicles of the Golgi network (3). Glycoprotein E is the major envelope protein of the WNV and is necessary for viral entry into the membrane of a cell. The protein undergoes

a conformational rearrangement triggered by low pH that results in a class II fusion event, which is required for viral entry. (4) In this study, we believe that successful integration of these antigens of interest into a vaccine will provide ample immunological protection against the WNV by ensuring that the virus is identified and eliminated by the host immune response before the virus is allowed to gain access to host cells in order to replicate.

CLINICAL ASPECTS:

The incubation period of the virus is typically 3 to 14 days. WNV infection is asymptomatic in approximately 80% of individuals but can lead to West Nile Fever in roughly 20% of afflicted persons, with about 1 in 150 persons developing severe neurological diseases associated with the virus. (2) The severe diseases that can occur include various neuroinvasive diseases such as West Nile encephalitis or meningitis, and the symptoms include headache, high fever, neck stiffness, stupor, disorientation, coma, tremors, convulsions, muscle weakness, paralysis, and sometimes death. These serious illnesses can occur at any age, although people over the age of 50 and immunocompromised patients, such as those who have undergone transplant surgeries or have HIV, are at the most risk for developing the serious forms of the disease. Diagnosis of WNV can be determined by a number of different tests, including IgG antibody sero-conversion by ELISA analysis, neutralization assays, viral detection by RT-PCR assay, and virus isolation by cell culture. Treatment for WNV severe symptoms include hospitalization, IV fluids, respiratory support, and prevention of secondary infections. There is no vaccine available for humans. The main defense against the virus lies in preventive measures, such as raising awareness of the risks associated with exposure to

the virus. Vector control has also shown to be effective, such as careful monitoring and control of mosquito populations in areas where the virus is common.

PATHOPHYSIOLOGY OF WNV:

Rodent models have provided much valuable insight into the mechanisms of WNV dissemination and pathogenesis. (5) Following the initial skin perforation, the initial replication of the WNV is theorized to occur in skin Langerhans dendritic cells. The infected cells then travel to and seed draining lymph nodes, resulting in a primary viremia and subsequent infection of peripheral tissues such as the spleen and kidney. Most of the time, WNV is largely cleared from the serum and other peripheral organs after the first week, with the immunocompromised and elderly immune systems being the subset that experiences the secondary infections. Persistent infection and viremia have been detected in immunosuppressed patients for more than 60 days. The way that WNV crosses the blood brain barrier remains largely unknown, but there are many theories that have supporting evidence. It is known that tumor necrosis factor- α mediates changes in the endothelial cell permeability of the blood brain barrier (BBB), and this is a possible facilitation method that allows WNV access to the CNS. Additional mechanisms may contribute to WNV CNS infection, including (i) infection or passive transport through the endothelium or choroid plexus epithelial cells (6), (ii) infection of olfactory neurons and spread to the olfactory bulb (7) (iii) a “Trojan horse” mechanism in which the virus is transported by infected immune cells that traffic to the CNS (8), and (iv) direct axonal retrograde transport from infected peripheral neurons (9). Although the precise mechanisms of WNV CNS entry in humans require additional study, changes in cytokine levels that may modulate BBB permeability and infection of blood monocytes and

choroid plexus cells have been documented in animal models. Experiments with small-animal models have demonstrated that T Lymphocytes are essential to protection against WNV. (10) As expected, individuals with hematologic malignancies and impaired T-cell function have an increased risk of neuroinvasive WNV infection. Upon recognition of a WNV-infected cell that expresses class I MHC molecules, antigen-restricted cytotoxic T lymphocytes proliferate, release pro-inflammatory cytokines, and lyse cells directly through the delivery of perforin and granzymes A and B or via Fas-Fas ligand interactions. Although there is a lot to be learned about the defined immunity process against WNV, both humoral and cellular responses are likely necessary for protection against the virus. Specific neutralizing antibodies are generated at late times after primary WNV infection, and the development of high-titer neutralizing antibodies after vaccination directly correlates with the typical antibody levels after a challenge with the virus. (11, 12). Some authors also suggest that cell adhesion molecules may help to traffic West Nile virus-infected immune cells into the central nervous system. (13).

EPIDEMIOLOGY OF WNV

WNV is one of the most widely distributed of all arboviruses with an extensive distribution among both hemispheres of the world. Many consider the origin of WNV to be North Africa, although the virus is now considered endemic in several places including Europe, South America, and even North America. (14) Since the discovery of WNV, infrequent human outbreaks were most commonly reported in groups of children, soldiers, and healthy adults in Israel and Africa. These outbreaks resulted in only minor illness in the majority of patients, and the few deaths that occurred were mainly

associated with old age. Since the mid-90s, however, severe virus infection associated with encephalitis have occurred in North Africa, Tunisia, Sudan, Europe, Russia, Israel, North America, Canada, and India. Among these, the mortality associated with the virus was at approximately 10% and occurred mainly in elderly patients. (14) There are no known predispositions to infection by WNV due to race or sex.

VACCINATION METHODS:

There currently is no vaccine for WNV, although some have been attempted and are awaiting further trials, mostly in animal models. A recombinant influenza virus expressing Domain III (DIII) of the WNV E protein has been identified as a vaccine candidate in models of mice. The WNV DIII was cloned into the N-terminal region of the influenza virus, rendering the functional activity of the influenza protein inactive. This study has found results of high IgG antibody ELISA titers through a subcutaneous injection of the vaccine in mice. (22) Schepp-Berglind et al. created and characterized an adenoviral vaccine vector (CAAdVAX-WNVII) which expressed four WNV proteins: C, prM, E, and NSI. Serum samples from mice that were administered this vaccine showed antibodies that neutralized lineage I and II viruses, and increased T-cell activity against WNV antigens were observed in vitro. (23) The main purpose of this study was to create a DNA vaccine and recombinant VZV (rVZV) vaccine expressing the PrM and gE antigens of the WNV.

DNA vaccination is a popular and proven method of vaccination against various diseases. It is accomplished by injecting cells with genetically engineering DNA so that the cells directly produce an antigen of that disease, producing a protective

immunological response from one's body. For many years, general vaccination has been accomplished by either directly introducing antigens that are characteristic of the virus, or by injecting a live, attenuated virus into the individual in order to prime their immune system. The DNA vaccine offers many different advantages over traditional vaccination methods. For example, DNA vaccines stimulate both the T- and B-cell responses, improved vaccine stability. It also is able to accomplish these things with the absence of infectious agents. Plus, they are relatively cheap and easy to mass produce. (15) The field of DNA vaccination is fast growing, with many vaccines already in use and many more currently in clinical trials. DNA vaccination is one of the methods which was researched and applied in this experiment, and it was done by inserting the Pre-membrane and glycoprotein E genes of the West Nile Virus into the vaccine vector pVAX1.

Another type of vaccination strategy that was utilized in the research conducted is the recombinant Varicella Zoster Virus (rVZV) vaccine, which also had the Pre-membrane and glycoprotein E antigens inserted into it. VZV is a virus which can result in chickenpox, primarily in younger patients, and can also reside as a dormant virus and later manifest into the shingles disease in older adults. This virus infection has been successfully prevented by the highly effective VZV vaccine that is readily available. Several researchers, including Dr. Wayne Gray, have studied this vaccine construct extensively and believe that it can be utilized in several other vaccination methods, such as HIV and several other viruses in the flavivirus family (16). He, among other researchers, cite the vaccine's safety and effectiveness, claiming it provides support for recombinant VZV (rVZV) vaccines to induce immunity against not only VZV but also against other pathogens. The ability of VZV vaccines to safely induce long-lasting

humoral and cellular immune responses provides advantages over other live, attenuated vaccine vectors and over killed and subunit vaccines. (16) There are also several other reasons that the vaccine can offer these advantages, such as the size of the vaccine (125kb) allows the stable insertion of several heterologous genes into specific loci without hindering viral replication. (17) Also, VZV replicates in the cell nucleus, which gives the opportunity for foreign, inserted genes to be spliced and replicated as is done in natural viral replication. (18) The host range of VZV is also limited to humans, eliminating the possibility of uncontrollable environmental spread. In addition, periodic subclinical reactivation of VZV from latency may provide re-stimulation of immune responses to VZV and foreign antigens (19). Experiments performed by Dr. Gray and his colleagues have shown promise in utilizing the rVZV vaccine to guard against SIV and other flaviviruses, including Dengue Fever and the re-emerging Zika Virus. The goal of this experiment is to create and characterize a rVZV vaccine that has the potential to induce an immune response to the WNV antigens discussed above.

CHAPTER II: DNA VACCINE CONSTRUCTION & TRANSFECTION

INTRODUCTION

DNA vaccines are a type of nucleic acid vaccine that is a relatively new and fast-growing discipline in the world of vaccines. They are accomplished by inserting various antigens of the disease inside of a vaccine construct, which then stimulates the immune system response. There are many advantages to using a DNA vaccine. They have been proven to be very safe, since no infectious agent is actually introduced to the body, such as the case with traditional vaccination methods which use live, attenuated viruses or killed viruses. They are also very easy to mass produce, relatively cheap to make, and have proven in some studies to be more stable than other vaccination strategies (15). DNA vaccines also stimulate both the T-cell and B-cell responses, activating both the cell-mediated and humoral immune response. Priming the immune system in both ways is key to successful protection against the infectious agents.

As effective as DNA vaccines can be, there are also disadvantages to be addressed, with several limiting factors to the potential wide-spread use of DNA vaccines. For one, this type of vaccination would be limited to pathogens that possess a distinct protein immunogen (molecules that directly trigger an immune response). There also is some speculation that introducing the foreign DNA into the body could have some kind of effect on a cell's normal protein expressing pathways, although this has yet to be shown as a major area of concern.

SPECIFIC AIM OF THIS STUDY:

The DNA vaccine vector used in this study is the pVAX1 vector (Thermo-Fisher Scientific) (*Figure 2*), which is Kanamycin resistant and is designed specifically for the construction of DNA vaccines due to the size of the vector and the availability of unique cloning sites in the MCS. The type of restriction site that is important in this study are the BamH1 sites, which is where the WNVprMgE gene will be inserted once they are digested (*Figure 3*). The gene of interest (WNVprMgE) will be inserted where the 5' primer end is downstream of the CMV promotor region of the pVAX1 plasmid (*Figure 4*). After the vaccine is created, successful expression of the gene of interest will be analyzed via PCR and further demonstrated through immunofluorescence of transfected Vero cells.

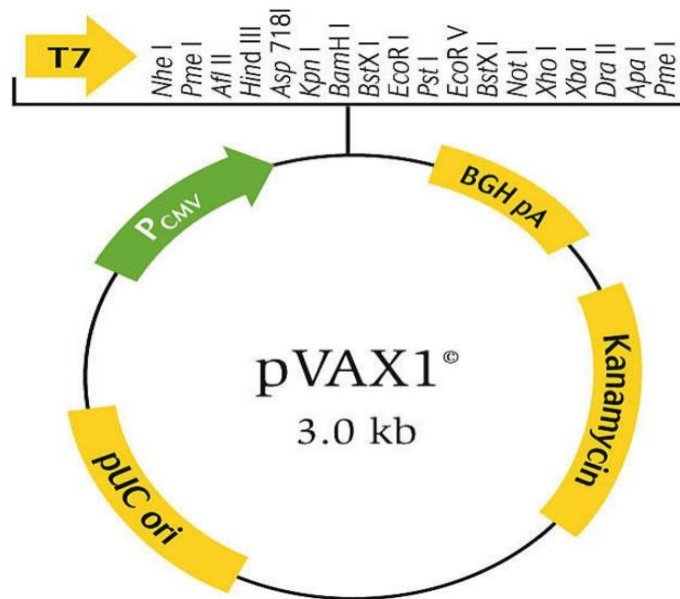


Figure 2 – pVAX1 structure

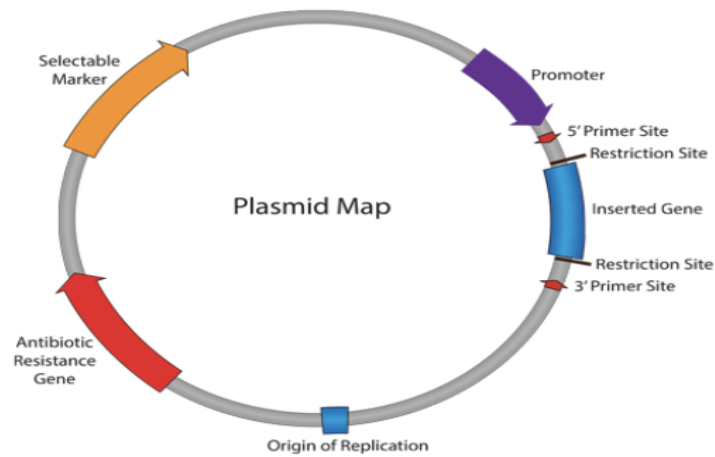


Figure 3 - Generic Plasmid Map

PROJECT OVERVIEW:

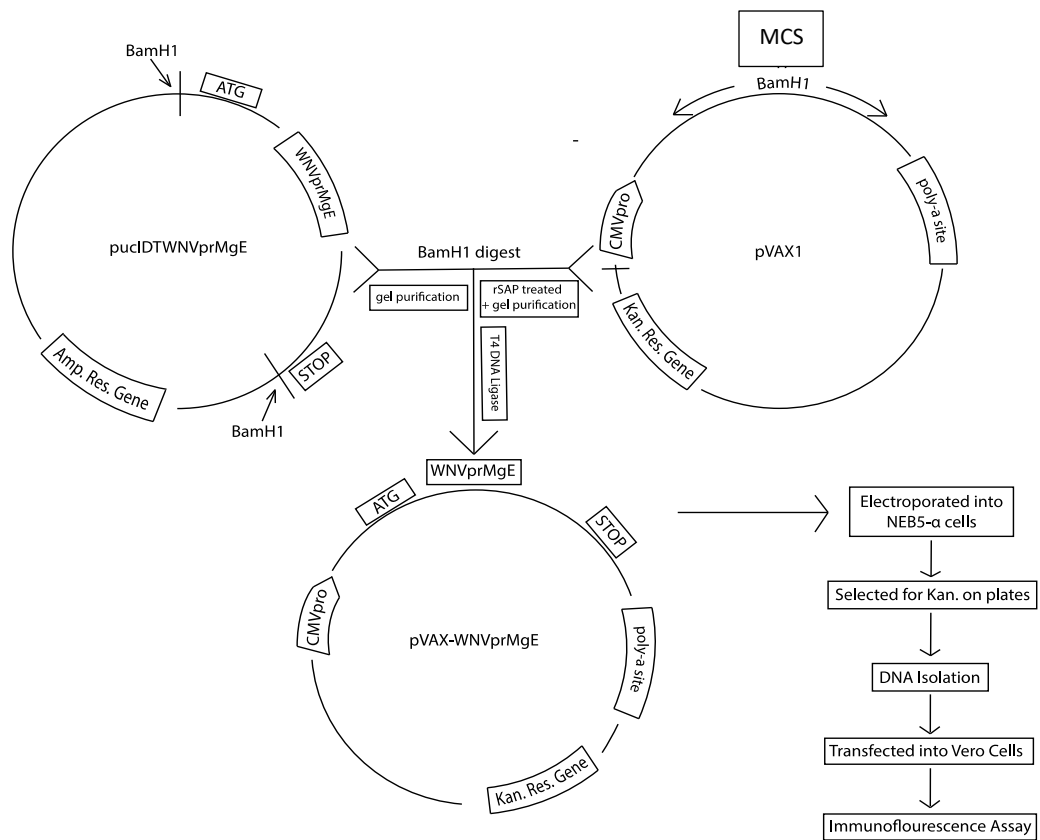


Figure 4 – Overview of DNA Vaccine Creation

In this study, we isolated the WNVprMgE gene from the pucIDT vector, cloned it into the pVAX1 vector, and then transfected the recombinant DNA into Vero cells to see if the WNVprMgE antigens would be expressed in the cells (*Figure 4*). The first obstacle that had to be overcome in the creation of this DNA vaccine was to separate the gene of interest, in this case the WNVprMgE, from its original vector, the pucIDT vector, so that it could be cloned into the new vaccine construct. The pucIDT vector contains BamH1 restriction sites where the genome can be cut with the restriction enzyme BamH1 during a digest, thus separating the WNVprMgE gene so that it could be cut out, isolated, and ligated into the new vector. The pVAX1 vector is the DNA vaccine into which the WNVprMgE gene was cloned. It also contains BamH1 restriction sites in the Multiple Cloning Site (MCS), which were digested using BamH1 to allow the insertion of the WNVprMgE. The MCS is a region commonly found in many engineered plasmids that can contain up to 20 different restriction sites, allowing diverse options for inserting various genes. Afterwards, a T4 DNA ligation protocol was followed in order to clone the WNVprMgE into the MCS region of the pVAX1 vector. Correct orientation of the cloned-in WNVprMgE was analyzed with PCR using forward and reverse primers to ensure that the WNV gene was inserted so that the ATG start codon is downstream of the CMV promoter of the pVAX1 plasmid, and sequencing results confirmed correct orientation (Eurofins Genomics). After the vaccine was created, the DNA was isolated and transfected into Vero cells, and expression of the WNV antigens in the Vero cells was confirmed with an immunofluorescence assay utilizing primary and secondary antibodies (Invitrogen). The primary antibody was a mouse anti-WNVprMgE, which means it will bind to the cells expressing the WNV antigens with the Fab end. The

secondary antibody was goat anti-mouse IgG-FITC, meaning they will bind to the to the Fc end of the primary antibodies. The secondary antibody was also fitted with a FITC fluorescent marker on their FC end, which makes them visible under a fluorescent microscope.

METHODS:

Midiprep DNA Extraction of pucIDT WNVprMgE from *E. Coli* (JM109)

E. Coli containing the pucIDT-WNVprMgE was grown overnight at 37 degrees in 5ml of Luric Broth (LB) + Ampicillin (Amp). The 5ml culture of pucIDT WNVprMgE was then transferred into 100ml of LB + Amp, which was incubated overnight at 37 degrees C. The DNA was extracted using a Qiagen Plasmid Midi Kit and precipitated overnight at 4 degrees C in isopropanol (IPA). The DNA was then spun down in a centrifuge, washed, and eluted into 50 µl of TE for storage. 5 µl of DNA was combined with 10 µl of loading dye, and this was run on a 0.5% agarose + Ethidium Bromide (EtBr) gel.

Restriction Digest and Gel Purification of WNVprMgE and pVAX1 with BamH1:

20 µl of 59.9 ng/µl WNVprMgE and 20 µl of 60.2 ng/µl pVAX1 was cut overnight using BamH1 and incubated in a water bath at 37 degrees C. The digests were set up as follows: pVAX1 vector: 20 µl of DNA, 1 µl of d2H2O, 1.5 µl of BamH1, 2.5 µl of 10X Buffer. WNVprMgE insert: 6 µl of DNA, 15 µl of d2H2O, 1.5 µl of BamH1, and 2.5 µl of 10X Buffer. One µl of Shrimp Alkaline Phosphatase (rSAP) was added to the pVAX1 vector in order to prevent re-ligation to itself and incubated for 1 hour at 37 degrees C in

a water bath. After the digest, the rSAP was heat-inactivated by incubation in a water bath at 65 degrees C for approximately 10 minutes. The digests were run on a 0.5% agarose + EtBr gel and gel-purified via a Quiagen Gel Extraction Kit, and the DNA was eluted into 30 µl of TE. The purified and digested DNAs were run on a 0.5% agarose + EtBr gel at 100V for 45 minutes for confirmation purposes. They were loaded into the gel wells as follows: WNVprMgE insert: 5 µl of sample DNA and 5 µl of loading dye.
pVAX1 vector: 5 µl of sample DNA and 5 µl of loading dye.

T4 DNA Ligation of pVAX-1 & WNVprMgE and Transformation:

A T4 DNA ligase reaction was set up as follows: 4 µl of pVAX-1 gel-purified DNA, 4 µl of WNVprMgE gel-purified DNA, 2 µl of Buffer 10X T4 Ligase buffer, and 1 µl of T4 Ligase. This reaction was incubated at room temperature for 1 hour. The ligase was incubated for 10 minutes in a water bath at 65 degrees C. The pVAX1-WNVprMgE DNA was electroporated into electro-competent NEB5-α cells using a 0.2 cm cuvet at 2.5 kilovolts. The pVAX1-WNVprMgE was then pipetted into 1ml of SOC media and the cells were then allowed to recover for an hour at 37 degrees C. The cells were plated on LB-Kanamycin plates and incubated overnight at 37 degrees C to grow clones. Clones from the plates were randomly picked onto fresh LB-Kan. plates and incubated over the weekend at 37 degrees C.

PCR of pVAX1-WNVprMgE:

Miniprep DNA isolations were performed for the selected clones. A PCR was set up as follows: 5 µl of DNA pVAX1-WNVprMgE NEB5-α, 1 µl of forward WNVprMgE extension primer, 1 µl of reverse WNVprMgE primer, 43 µl of PCR Super-mix, for a total of a 50-µl reaction. The PCR reaction was carried out as follows: (30x) 2-minute initial denature at 94 degrees C, 45 second denature at 94 degrees C, 1 minute anneal at 55 degrees C, and a 2-minute elongation at 72 degrees C. This was followed by a 7-minute final elongation at 72 degrees C and indefinite incubation at 4 degrees C. The primers used were: Forward WNVprMgE = GCCACCATGGGAGGAAGACC. Reverse WNVprMgE = CTATTAAGCGTGCACGTTACGG (reverse compliment). The PCR product was run on a 0.5% Agarose + EtBr gel for 60 minutes at 100V. The gel wells were loaded as follows: well 1 (MW) = 3 µl of DNA Ladder and 5 µl of loading dye. Well 2 (PCR product): 10 µl of DNA and 5 µl of loading dye.

DNA Extraction and BamH1 digest of pVAX1-WNVprMgE:

Six clones were selected at random and inoculated into a 5ml culture tube of LB and 10 µl of Kanamycin (clones 5-10), where they were incubated overnight at 37 degrees C. DNA was extracted from the clones using the Qiagen Mini-Prep kit for confirmation of pVAX1 and WNVprMgE DNA. The DNA was eluted into 50 µl of TE. A digest reaction was set up for 3 hours at 37 degrees C as follows: 5 µl of DNA, 16 µl of d2H2O, 1.5 µl of BamH1, and 2.5 µl of Buffer 10X. The digested product was then run on a 0.5% Agarose + EtBr gel at 100V for 45 minutes and analyzed.

PCR confirmation of pVAX1-WNVprMgE clones 5-8:

A PCR was set up for each of the pVAX1-WNVprMgE NEB5- α positive clones (5, 6, 7, and 8) as follows: 5 μ l of clone DNA, 1 μ l of reverse WNV-prMgE primer, 1 μ l of T7 promoter, and 43 μ l of PCR Super Mix. The PCR parameters were as follows: (30x) 2 minutes initial denature at 94 degrees C, 45 second denature at 94 degrees C, 1 minute anneal at 55 degrees C, 3-minute elongation at 72 degrees C. A final elongation occurs at 72 degrees C for 7 minutes, followed by indefinite incubation at 4 degrees C. The PCR products (clones 5, 6, 7, and 8) were then run on a 0.5% Agarose + EtBr gel for 45 minutes at 100V for confirmation as follows: well #1 – 3 μ l DNA Ladder and 5 μ l loading dye. Wells #2-5 – 10 μ l of PCA product and 5 μ l of loading dye. (*Figure 9*)

Midiprep DNA Extraction of pVAX1-WNVprMgE NEB5- α (clone 5):

Five ml of LB broth and 10 μ l of Kanamycin were inoculated with a positive clone of pVAX1-WNVprMgE NEB5- α and was allowed to incubate overnight in a culture tube at 37 degrees C. This culture was then combined with a beaker containing 100ml of LB broth and 200 μ l of Kanamycin. This beaker was allowed to shake and incubate overnight at 37 degrees C. The DNA was extracted using a Quiagen Plasmid Midi Kit (25) and was eluted into 100 μ l of EB Buffer and stored.

BamH1 Digest of pVAX1-WNVprMgE NEB5- α (clone 5):

A digest of the positive pVAX1-WNVprMgE NEB5- α clone was set up as follows: 1.5 μ l of BamH1, 2.5 μ l of Buffer 10X, 2 μ l of DNA, and 19 μ l of d2H2O for a total reaction of 25 μ l. This digest was allowed to incubate overnight in a water bath @ 37 degrees C. The

digested DNA was then run and analyzed on a 0.5% Agarose +EtBr gel at 100V for 45 minutes.

Transfection of Vero Cells with pVAX1-WNVprMgE

The Vero cells were seeded onto culture chamber slides with a $10^5 + 2 \times 10^5$ per chamber. The Lipofectamine 3000 Reagent (0.5 + 1.0 μ l) was diluted in Opti-MEM Medium (2 x 30 μ l) and mixed. The Master Mix was prepared as follows: dilute 0.5 ng DNA in 60 μ l of Opti-MEM medium and 1 μ l of P3000 was added and mixed well. 30 μ l of the diluted DNA was added to each tube of diluted Lipofectamine 3000 Reagent (1:1 ratio) and mixed well. This was incubated for 5 minutes at room temperature. The DNA-lipid complex (60 μ l each) was added to the Vero cells. The cells were then incubated for 2-4 days.

Immunofluorescence Assay of Transfected Vero Cells with p-VAX-WNVprMgE positive clone (clone 5):

250 μ l of methanol/acetone was added to each chamber of cells and incubated for 10 minutes at room temperature. The methanol/acetone was removed and 250 μ l of 1% BSA was added to each chamber and incubated for 30 minutes at room temperature. The primary antibody (mouse Anti-WNVgE mAb E34, Invitrogen) was diluted in 1% BSA in a 1:200 ratio and was added to both of the control chambers (200 μ l/chamber). This was allowed to incubate at room temperature for 2 hours. The primary antibody was then removed and the chambers were washed 4 times with 1X PBS. 100 μ l of the secondary antibody (goat - Anti-mouse IgG FITC labeled, Invitrogen) was added to the chambers

and was allowed to incubate for 2 hours in the dark. The secondary antibody was then removed and the chambers were washed 4 times with 1X PBS. The slides were then analyzed using a fluorescent microscope to check for successful expression of the WNVprMgE antigens in the infected cells.

RESULTS

A midiprep DNA extraction from inoculated *E. Coli* bacteria (JM109) was performed in order to maximize the pucIDT-WNVprMgE DNA concentration. The WNVprMgE was originally cloned into the pucIDT vector, so digestion of the BamH1 restriction sites and separation of the DNA by gel-electrophoresis, which sorts molecules by molecular weight, is necessary to isolate the gene of interest (WNVprMgE). The DNA was cut with BamH1 enzyme, and 50 μ ls (μ l) of the digest was run on an agarose gel (*Figure 5*). The size of the pucIDT vector is 2.7kb, while the size of the WNVprMgE is 2.1kb. Good separation of the pucIDT and WNVprMgE DNA was not achieved on this first attempt, but better results were achieved later by optimizing the agarose concentration of the gel.

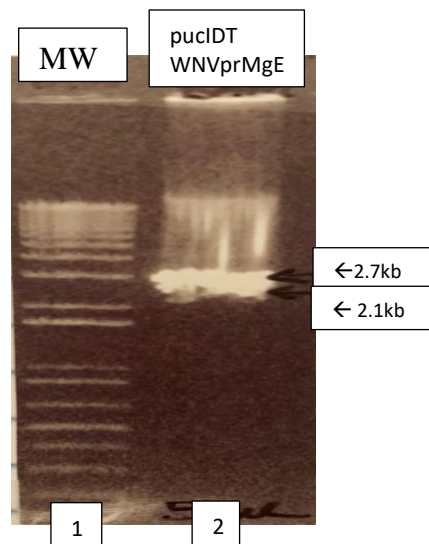


Figure 5 - UV image of digested pucIDT WNVprMgE

The pVAX1 and WNVprMgE DNAs were digested with the enzyme BamH1. Gel purification is performed in order to minimize possible contamination of both DNAs so that a successful ligation can be performed. It is also conducted primarily so that the WNVprMgE gene can be successfully removed from its original vector (pucIDT). Prior to the gel purification, the pVAX1 DNA was treated with a small amount of Shrimp Alkaline Phosphatase (rSAP) in order to prevent re-ligation of the vector to itself, which would prevent insertion of the WNVprMgE gene during the ligation. The gel electrophoresis was run out at a lower voltage for a longer period of time in order to create as much separation between the two bands as possible in order to minimize contamination of the WNVprMgE DNA (third lane of *Figure 6*). The bottom band in lane three contains the WNVprMgE DNA and it was excised carefully with a sterile scalpel so that the purified pVAX1 and purified WNVprMgE DNA could be ligated together. DNA ligation occurs with the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in the DNAs that are being joined. Essentially, it

facilitates the joining of the two sets of DNAs together so that they can both be present within a single plasmid.

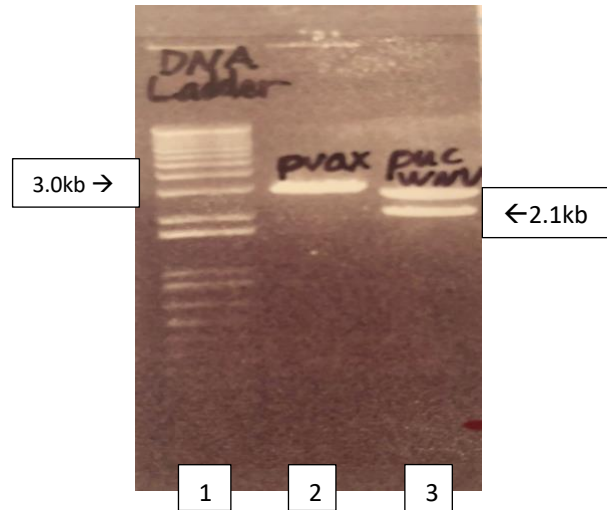


Figure 6 - UV image of Restriction Digest & gel purification of WNVprMgE & pVAX1 DNA

The ligated pVAX1-WNVprMgE DNA was then electroporated into electro-competent *E. Coli* NEB5- α cells, which were plated onto LB Amp. plates to grow up clones. DNA was isolated from the clones at random in order to re-analyze the DNA and verify that the WNVprMgE DNA is present. WNVprMgE forward and reverse primers were used to amplify the WNV gene, which would confirm its presence. The PCR product of clone #1 is observed in the second lane of the gel image of *Figure 7*, while the first lane is the molecular marker. The bottom band in lane 2 is approximately 2.1kb, which is the expected size of the WNVprMgE DNA, indicated its presence. The streaking observed in the UV image could be due to possible contamination of some of the DNA, overloading of the lane, or could be due to the fact that this gel was only run for 45 minutes at 100V; a longer run time could possibly produce better, cleaner separation of

the bands. The important conclusion of this image is confirmation of the fact that the WNVprMgE DNA was present after PCR, which amplifies the gene that was primed.

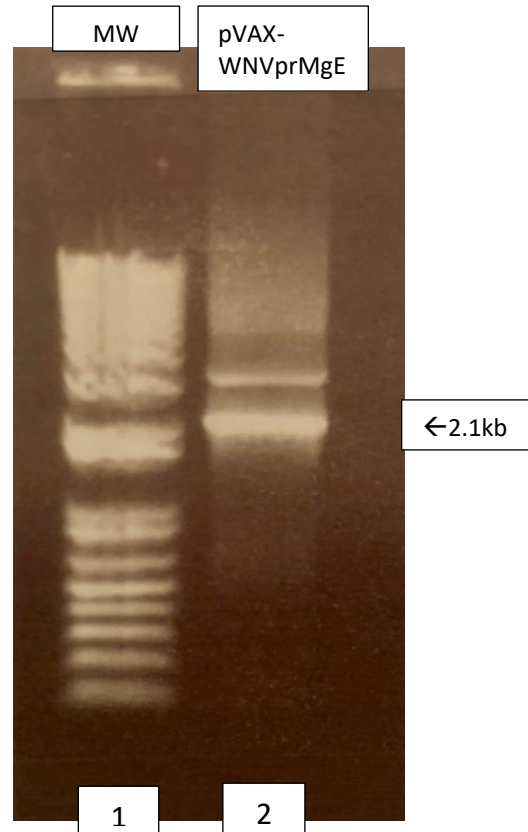


Figure 7 - UV image of PCR product pVAX1-WNVprMgE in NEB5- α cells

The next step to be done is to confirm the correct orientation of the gene. The results with the original clones were ultimately negative, because even though the insert DNA was present, it did not clone in the correct orientation where the WNVprMgE gene ATG start codon is downstream of the CMV promotor. Further clones were randomly selected and the process was repeated in hopes of finding clones where the insert was present in the vector in the correct orientation (*Figure 8*).

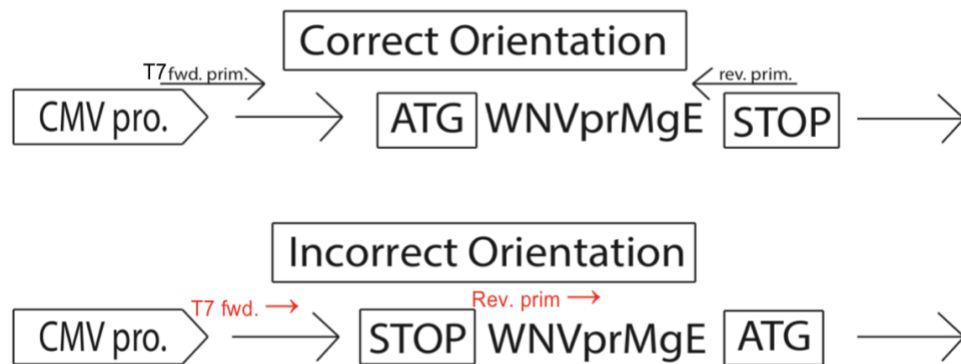


Figure 8 - PCR Orientation

Six more clones were randomly selected from the previous plate, and were inoculated and incubated overnight, and then DNA was extracted from each using a Miniprep Kit for analysis. The DNA was digested using BamH1 and the digested product of clones 5-9 were run on a 0.5% agarose + EtBr gel (*Figure 9*). All clones were observed to be positive in the sense that both the pVAX1 vector and WNV insert DNA were present. A second PCR was then conducted for each of the new clones to confirm if any had the WNVprMgE gene insert cloned in in the correct orientation.

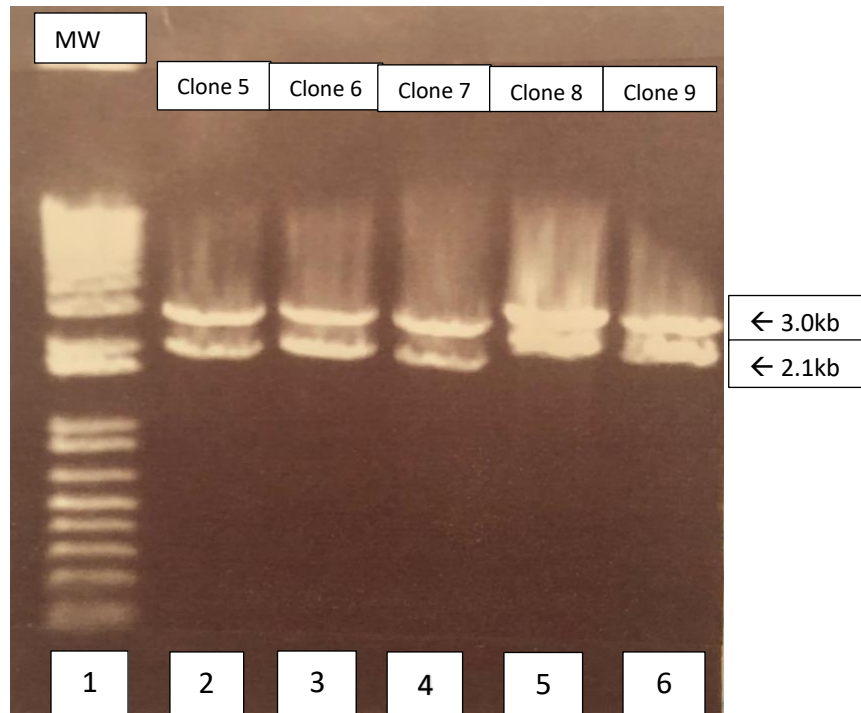


Figure 9 - UV image of digested pVAX1-WNVprMgE NEB5-α clones 5-9

The WNV gene's presence alone is not enough to ensure that it is a potentially viable vaccine. In order to determine if the gene of interest is being correctly *expressed* in the vaccine construct, Polymerase Chain Reaction (PCR) amplification is necessary. When the gene of interest is ligated into the pVAX1 construct, it can be cloned in either the correct or incorrect orientation due purely to chance. To determine if a clone has the WNV gene in the correct orientation, a forward T7 primer and reverse WNVprMgE primer were used to determine the orientation of the start and stop codons of the gene of interest relative to the CMV promotor and Polyadenylation site of the pVAX1 vector. The CMV promotor is used commonly for general expression and is a strong mammalian expression promotor isolated from the Human Cytomegalovirus. (24) The choice of promotor is very important because it needs to be suited to work well within the host organism, which is why the CMV promotor is used since the host

organism would be, preferably, a human. It is very important that the gene of interest is inserted in the correct orientation where the start codon (ATG) is relative to the CMV promotor so that the successful expression of the WNVprMgE is accomplished. The gene of interest also needs to be inserted where the 3' end, the end that is being terminated, is upstream of the polyadenylation site. Polyadenylation adds a transcription termination at the 3' end of the gene being expressed, so it is vital for successful transcription that the WNV gene is in the orientation where the stop codon is upstream to the polyadenylation site so that the process of releasing the newly synthesized RNA from the transcription machinery can begin. Confirmation of the gene of interest's orientation was analyzed via PCR using a T7 forward and WNVprMgE reverse primer. Primers enhance both strands that are of interest, in this case the WNVprMgE strands. The forward primer is for the beginning of the gene and the reverse primer begins the complimentary strand. By using both of these primers the gene's orientation can be determined by observing a 2.1kb band in the agarose gel, which would indicate that the gene was primed and amplified.

A PCR was conducted using a WNVprMgE reverse primer along with a T7 promotor. PCR products for clones 5, 6, 7, and 8 were analyzed on an agarose gel (*Figure 9*). Clones 5, 7, and 8 were observed to be positive for correct orientation of the WNVprMgE insert, indicated by a band being present at 2.1kb, while clone 6 was negative (*Figure 10*). Clones 5 and 7 were selected and sent for sequencing for further confirmation.

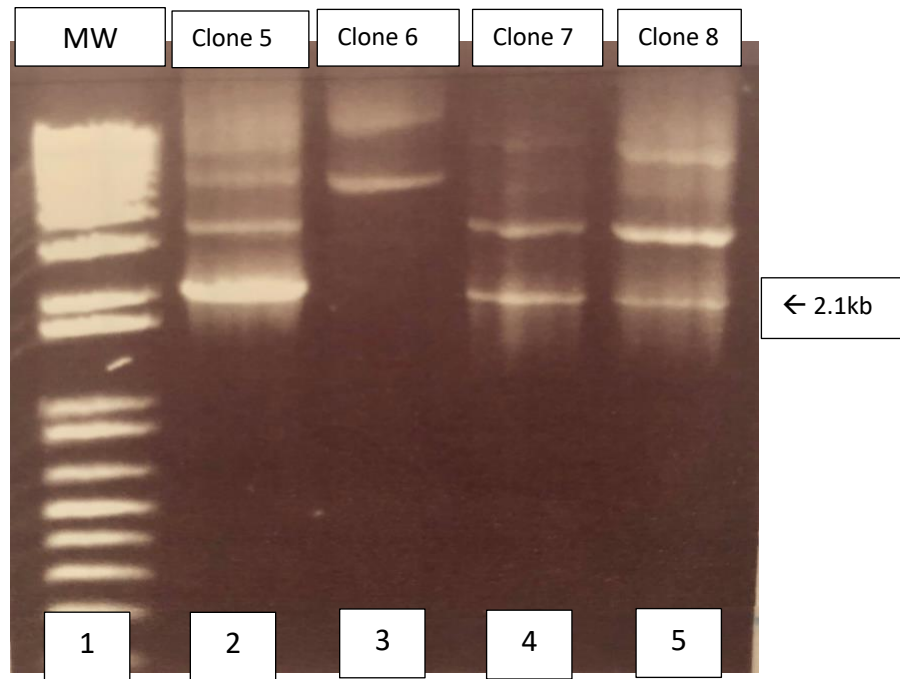


Figure 10 - PCR product of pVAX1-WNVprMgE clones 5-8

A digest of the pVAX1-WNVprMgE NEB5- α clone 5 was conducted for further confirmation while awaiting sequencing results (Eurofins Genomics). Sequencing data, when interpreted, can provide absolute confirmation of the presence of the start codon of the gene of interest relative to the CMV promotor of the plasmid. The UV image can be observed in *Figure 11*, with lane one being the molecular marker and lanes two and three containing the digested material that was divided evenly between the two lanes. The UV image further confirms the presence of both the insert and vector DNA. (Two lanes were used because of excess digest, both lanes contain digested pVAX1-WNVprMgE NEB5- α clone 5 DNA)

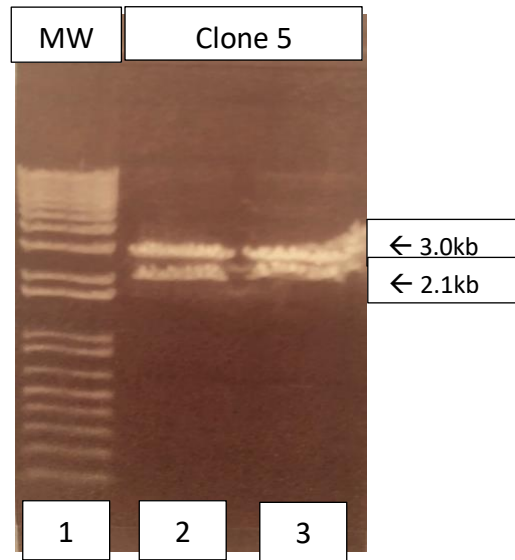


Figure 11 - BamHI Digest of pVAX1-WNVprMgE NEB5-α Clone #5

Transfection of pVAX1-WNVprMgE into Vero Cells:

Vero cells were seeded onto chamber slides and then transfected with the pVAX1-WNVprMgE isolated DNA, and confirmation of the successfully expressed antigens were analyzed via immunofluorescence. Primary and secondary antibodies were used in this assay (Invitrogen). The primary antibody attaches to the antigen (WNVprMgE) with its Fab end, and the secondary antibody attaches to the primary antibody's Fc end and contains a fluorescent marker for visual confirmation during the assay on its Fc end. Positive and negative controls were utilized in this study, with the negative controls receiving no amount of pVAX1-WNVprMgE DNA. All controls were given the primary and secondary antibody treatment. The chambers were analyzed under a fluorescent microscope. The fluorescent glow that can be observed in the assay confirms that the primary antibody was able to find and attach to the antigen, which means the antigenic proteins were successfully expressed in the Vero cells, indicating that the vaccine creation was a success. (*Figure 12*)

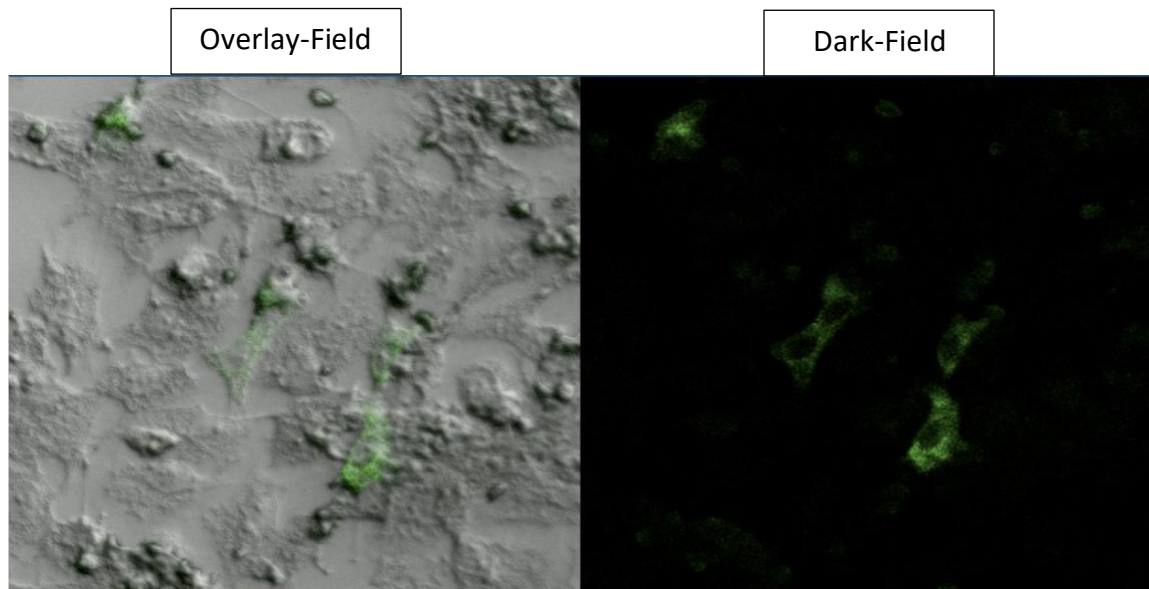


Figure 12 – Immunofluorescence Assay of Transfected Vero Cells

CONCLUSION:

The WNVprMgE gene was successfully cut and isolated from the pucIDT vector by the digestion of the BamH1 restriction sites. The same restriction sites in the pVAX1 vector were digested, and re-ligation of the vector to itself was prevented by the addition of rSAP, which cleaves the exposed 5' and 3' phosphate ends. Successful T4 DNA ligation was accomplished by inserting the gene of interest into the pVAX1 vector. After multiple attempts, correct orientation of the insert was confirmed in select clones via PCR amplification of the WNV gene using a T7 forward primer and reverse WNVprMgE primer. Sequencing results (Eurofins Genomics) offered further confirmation that the gene was inserted in the correct orientation. The pVAX1-WNVprMgE clone 5 DNA was isolated, and successful transfection of Vero cells was accomplished and observed via an immunofluorescence assay using primary and secondary antibodies, confirming

successful WNVprMgE expression, meaning the vaccine was created and that it was successful in expressing the WNV antigens in the Vero cells. The success of this experiment is important because we have proven that successful WNV antigen expression is possible in cells through the use of our vaccine, which could potentially be utilized as a method of preventing this disease which has killed thousands of people.

The next steps in this study would be to move on to animal trials using immunized mice; if it showed favorable results in animal trials, it could potentially move on to human trials. The pVAX1 vector is one of the main DNA vaccine vectors which has been approved for use in human trials, which is the primary reason it was used in this study. It is clear that a vaccine against WNV is needed, and I believe we are now a little closer to that goal.

CHAPTER III: RECOMBINANT VZV VACCINE CONSTRUCTION

INTRODUCTION:

As stated previously, the Varicella-zoster Virus (VZV) vaccine induces immunity against childhood chickenpox and shingles disease in older adults. (16) Due to many pros of the vaccine, the theory is that this vaccine might be altered to form a recombinant vaccine expressing the antigens of interest of various other diseases. The thought process behind the possible viable use of this vaccination strategy is due to its widespread use and safety, as well as the success rate of lifelong protection of those vaccinated against the Varicella Zoster Virus. The vaccine also replicates in the nucleus of the cell, which means that the inserted antigens would be spliced and replicated along with the virus in a very natural way. The purpose of this chapter is to discuss the preliminary methods of creating a recombinant Varicella Zoster Virus (rVZV) vaccine expressing the WNVprMgE antigens.

SPECIFIC AIM OF THIS STUDY:

The goal of this experiment is to insert the WNVprMgE into the VZV vaccine virus genome. The entire VZV genome is cloned into a bacterial artificial chromosome (BAC). In this project, the WNVprMgE gene was separated from the pucIDT vector and

successfully ligated into the MCS of the p-fast BAC vector. The CMV promotor, WNV gene, and polyadenylation site of the p-fast BAC vector were then transposed into the attachment (*att*) site of the engineered VZV BAC. Successful transposition resulted in the *lacZ* gene of the VZV BAC being disrupted, as determined through blue/white screening of clones on LB plates treated with Xgal. If white clones appear, then it proves that the *lacZ* site has been disrupted and that the WNVprMgE gene was successfully cloned into the VZV BAC.

PROJECT OVERVIEW:

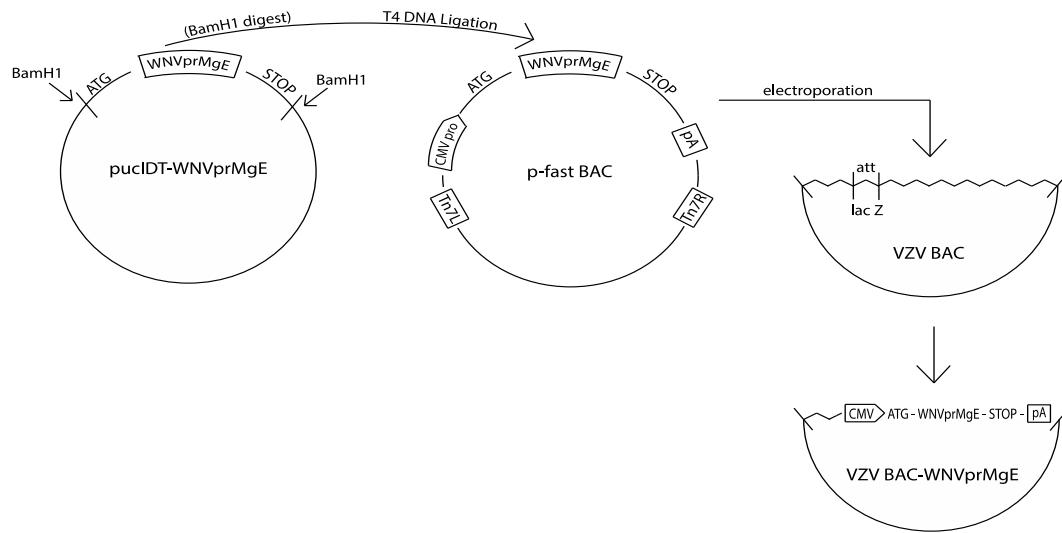


Figure 13 – Overview of rVZV Vaccine Creation

The first task to accomplish is identical to the creation of the DNA vaccine: the WNVprMgE DNA must be separated from the pucIDT vector (*Figure 13*). This is accomplished by digesting the BamH1 restriction sites, separating the molecules by

molecular weight via gel-electrophoresis, and excising the separated WNVprMgE DNA with a sterile scalpel. The first vector that the WNV gene must be cloned into is the p-fast BAC. The p-fast BAC also contains a BamH1 restriction site in the Multiple Cloning Site (MCS), which when digested, allows the WNVprMgE gene to be inserted (*Figure 13*). The p-fast BAC DNA was treated with 1 µl of Shrimp Alkaline Phosphatase (rSAP) after it was digested with BamH1 in order to prevent re-ligation to itself. After the WNVprMgE DNA was successfully cloned in to the p-fast BAC vector, the p-fast BAC-WNVprMgE DNA was isolated and electroporated into a BAC which contained the entire Varicella Zoster Virus (VZV) genome. During electroporation, everything between the Tn7R and Tn7L transposon sites of the p-fast BAC was translocated into the *att* site that is present in the VZV BAC, including the CMV promotor, the WNV gene, and the polyadenylation site (*Figure 13*). The *att* site allows insertion of DNA so that it will be incorporated into its genome and will replicate when the bacteria does. This *att* site was engineered to contain a lacZ gene, which will be disrupted when the WNVprMgE DNA is inserted. This mechanism allows us to conduct blue/white color screening of clones that are plated on LB plates treated with Xgal. If the WNV gene was successfully inserted into the *att* site, the lacZ gene would be disrupted and the clone would appear white on the plate and successful insertion of the WNV gene into the VZV BAC would have been accomplished. If the WNV gene was not successfully inserted, the lacZ site would not be disrupted and the clones appear blue on the plate.

METHODS:

Overnight digest of p-fast BAC and WNVprMgE with BamH1:

An overnight digest of p-fast BAC and pucIDT-WNVprMgE was set up as follows: (2x) 3 µl of DNA, 1.5 µl of BamH1, 2.5 µl of Buffer 10X, and 18 µl of d2H2O, for a 25-µl total reaction. The digests were allowed to incubate overnight in a water bath at 37 degrees C. The digests were run on a 0.5% Agarose + EtBr gel at 90V for 2 hours to create maximum DNA band separation.

Gel purification of p-fast BAC and WNVprMgE digests:

The p-fast BAC and WNVprMgE bands were excised from the 0.5% Agarose + EtBr gel using a clean scalpel. The DNAs were gel purified using the QIAquick gel extraction kit and eluted into 60 µl of TE for storage. A 5 µl sample of gel-purified DNA was run on a 0.5% Agarose + EtBr gel as follows: (2x) 5 µl of gel-purified DNA, 5 µl of loading dye, and 5 µl of d2H2O for a 15-µl total reaction.

T4 Ligation reaction of purified p-fast BAC and WNVprMgE:

1 µl of Shrimp Alkaline Phosphatase (rSAP) was added to the gel purified p-fast BAC in order to prevent re-ligation to itself, and was allowed to incubate in a water bath for 1 hour at 37 degrees C. The digest was then heat-inactivated at 65 degrees C for 10 minutes. The ligation reaction between the p-fast BAC and WNVprMgE was set up as follows: 10 µl of WNVprMgE, 7 µl pf p-fast BAC, 1 µl of T4 ligase, and 2 µl of 2X Buffer. This was allowed to incubate at room temperature for one hour.

Electroporation of p-fast BAC + WNVprMgE

7 µl of the ligated p-fast BAC-WNVprMgE DNA was electroporated into 53 µl of electro-competent NEB5- α cells and 5 µl of d2H2O using a 2cm cuvette. The cells were allowed to recover in 1ml of LB for 1 hour at 37 degrees C and 200 rotations per minute (RPM). The cells were then plated onto 3 LB + ampicillin (Amp.) plates and allowed to incubate over the weekend at 37 degrees C to grow clones. Many clones were picked at random onto another LB + Amp. plate and grown overnight at 37 degrees C.

Miniprep DNA Extraction of p-fast BAC + WNVprMgE NEB5- α clones:

Four clones were chosen at random and inoculated into 5 ml of LB broth + 10 µl of Amp and were grown overnight at 37 degrees C and 200 RPM. DNA was extracted from the clones using the QIAprep Miniprep Kit and eluted into 50 µl of TE for storage.

Overnight BamH1 digest of p-fast BAC + WNVprMgE clones (1-4):

Four overnight digests with BamH1 were set up as follows: (4x) 8 µl of clone DNA, 1.5 µl of BamH1, 2.5 µl of Buffer 10X, and 13 µl of d2H2O. The four digests were allowed to incubate overnight in a water bath at 37 degrees C and were run on a 0.5% Agarose + EtBr gel at 100V for one hour the next day. The results were analyzed via UV trans-illumination. Clones 1 and 3 appeared to be positive for the presence of both the p-fast BAC and WNVprMgE DNAs and were selected to be analyzed further via PCR.

PCR confirmation of p-fast BAC + WNVprMgE positive clones (clones 1 & 3)

A PCR was set up using the fwd. and rev. primer of the WNVprMgE and was allowed to run overnight as follows: Clones #1: 1 µl of fwd. WNVprMgE primer, 1 µl of rev.

WNVprMgE primer, 4 µl of digested DNA, and 44 µl of PCR Super Mix. Clone #3: 1 µl of fwd. WNVprMgE primer, 1 µl of rev. WNVprMgE primer, 4 µl of digested DNA, and 44 µl of PCR Super Mix. The PCR was carried out as normal protocol, except with a 4-minute elongation step. The PCR products were analyzed on a 0.5% Agarose + EtBr gel at 90V for one hour and analyzed using UV trans-illumination.

PCR reaction of p-fast BAC + WNVprMgE clones (1 & 3) with positive and negative controls:

A PCR for the positive p-fast BAC-WNVprMgE clones was set up as follows: Clone #1: 1 µl of fwd. WNVprMgE primer, 1 µl of rev. WNVprMgE primer, 4 µl of digested DNA, and 44 µl of PCR Super Mix. Clone #3: 1 µl of fwd. WNVprMgE primer, 1 µl of rev. WNVprMgE primer, 4 µl of digested DNA, and 44 µl of PCR Super Mix. Positive control: 4 µl of pucIDT WNVprMgE DNA, 1 µl of fwd. WNVprMgE primer, 1 µl of rev. WNVprMgE, and 44 µl of PCR Super Mix. Negative control: 4 µl of p-fast BAC DNA, 1 µl of rev. WNVprMgE primer, 1 µl of fwd. WNVprMgE primer, and 44 µl of PCR Super Mix. The total reaction volume for each was 50 µl. The PCR was carried out using normal protocol, except with a 2-minute elongation step. The PCR products were run on a 0.5% Agarose + EtBr gel at 90V for 1 hour and analyzed using UV trans-illumination to confirm presence of the primed WNVprMgE gene.

Midiprep and Transposition of WNVprMgE into VZV BAC LacZ

The DNA from clone 3 was extracted and eluted into 200 µl of TE via the Qiagen Plasmid Midi Kit protocol for storage. (Midiprep concentration = 1550 ng/µl) A BamHI

digest was set up as follows: 2 µl of DNA, 1.5 µl of BamH1, 2.5 µl of Buffer 10X, and 19 µl of d2H2O for a 25 µl total reaction. This was allowed to incubate overnight in a water bath at 37 degrees C. The digest were run on a 0.5% Agarose + EtBr gel and analyzed via UV-trans-illumination for further confirmation of the p-fast BAC and WNVprMgE presence. The p-fast BAC + WNVprMgE clone 3 was then electroporated into *E. coli* containing the VZV BAC + LacZ-att Tn7 (9/20/17) using a 0.1cm cuvette as follows: 50 µl of the BC VZV BAC + LacZ-att. Tn7 NEB5-α clone 4.1, 10 µl of d2H2O, 1.5 µl of pMON 7124 DNA, and 1 µl of p-fast BAC + WNVprMgE clone 3. This was then re-suspended in 1ml of LB and allowed to recover for 2 hours. This process was repeated using a negative control, which uses d2H2O in place of the DNA. The cells were plated on LB + CAM/TET/AMP plates for triple antibiotic screening. White clones were then selected and picked onto a separate plate, and 3 clones were chosen at random and inoculated into 5ml LB and incubated overnight. These cultures were then combined with 100 µl of LB and allowed to incubate overnight.

RESULTS

Isolation of WNVprMgE DNA & Digestion of p-fast BAC vector

The WNVprMgE DNA separation from the pucIDT vector was accomplished by digestion of the BamH1 restriction sites of the pucIDT, separation of the DNAs by molecular weight via gel electrophoresis, and excision of the WNVprMgE DNA band with a sterile scalpel. The p-fast BAC DNA was also digested with BamH1 and treated with a small amount of Shrimp Alkaline Phosphatase (rSAP) to prevent re-ligation of the vector to itself, which would prevent the WNVprMgE gene from being ligated into the p-fast BAC vector.

Gel-purification of the p-fast BAC & WNVprMgE DNAs

The p-fast BAC and WNVprMgE DNAs were gel purified before the ligation in order to remove any possible contamination. The gel-purified p-fast BAC and WNVprMgE DNA can be observed in *Figure 14* in the second and third lanes, respectively. This gel purification was done in order to limit the risk of DNA contamination.

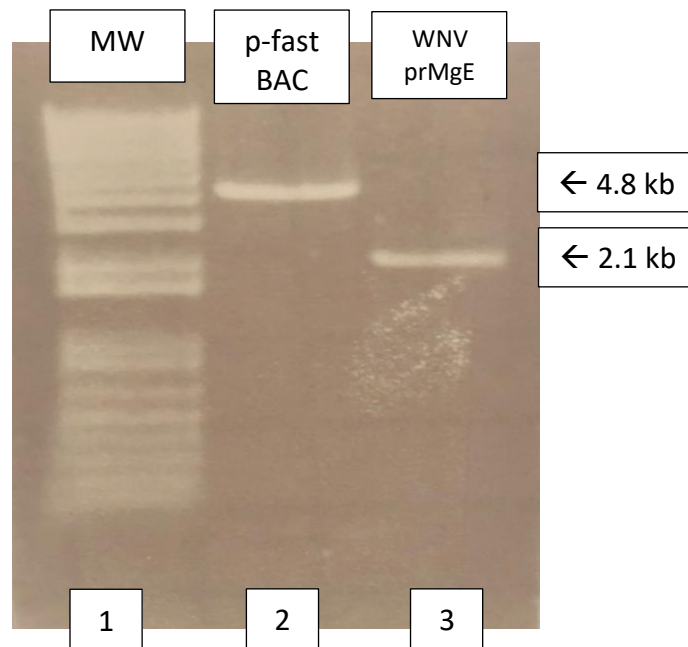


Figure 14 - BamH1 Digest + gel purification of p-fastBAC & WNVprMgE

p-fast BAC & WNVprMgE Ligation & Electroporation into NEB5- α cells

The p-fast BAC and WNVprMgE DNAs were ligated together using a T4 DNA ligation protocol. The ligated p-fast BAC-WNVprMgE DNA was electroporated into electro-competent NEB5- α cells and clones were grown over the weekend against Amp. selection to help avoid possible contamination.

BamH1 Digest of p-fast BAC-WNVprMgE clones

DNA was isolated from the clones and cut with BamH1, and the digests were analyzed on the UV image seen in *Figure 15*, where the first lane contains the molecular weight marker and lanes 2-5 contain clones 1- 4. Clones 1 and 3 appear to be positive, meaning they have the presence of both the p-fast BAC and WNVprMgE DNA, and were further analyzed via PCR.

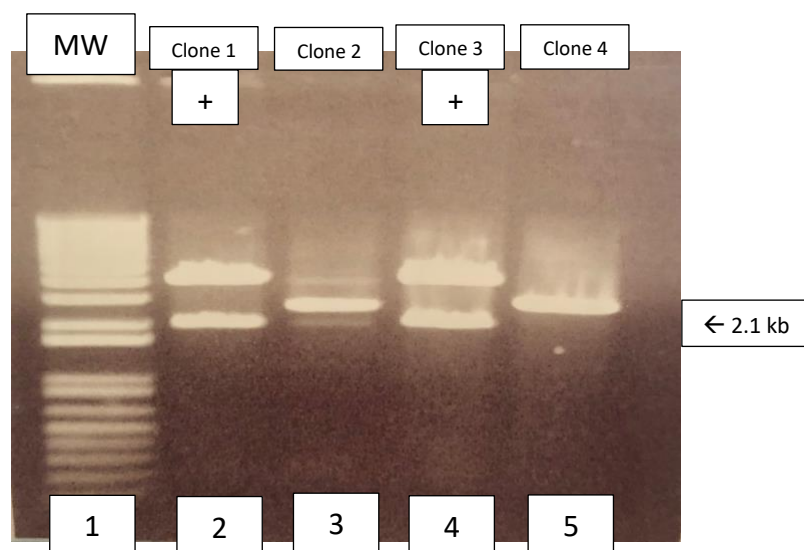


Figure 15 - BamHI Digests of p-fastBAC + WNVprMgE NEB5-α clones 1-4

PCR of p-fast BAC-WNVprMgE with Fwd. and Rev. Primers

Clones 1 and 3 were selected, and four 50 µl reactions were carried out (2 reactions per clone) and amplified via PCR, which can be observed in *Figure 16*. Every clone received the p-fast BAC forward primer, while one reaction for each clone received the WNVprMgE forward primer and the other received the WNVprMgE reverse primer. Orientation of the WNVprMgE gene was analyzed by sequencing results (Eurofins Genomics). The ATG start codon of the WNVprMgE gene was found to be downstream of the CMV promotor of the p-fast BAC vector, inferring correct orientation of the inserted gene.

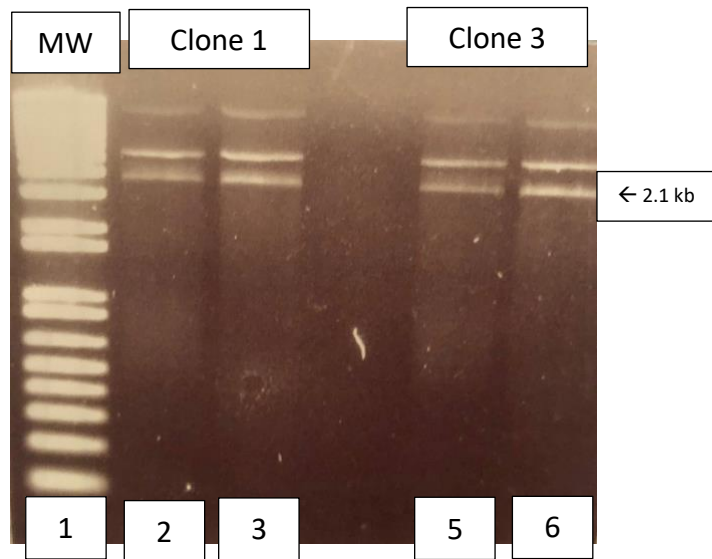


Figure 16 - UV image of PCR product of p-fast BAC + WNVprMgE clones 1 & 3

Four more PCRs were conducted, this time with a two-minute elongation step, and the results can be observed in *Figure 17*. All four reactions were run with a forward and reverse primer for the WNVprMgE. The first lane is the molecular weight solution, the second lane contains clone 1, the third lane contains clone 3, the fourth lane contains the positive control, and the fifth lane contains the negative control. The positive control contained pucIDT WNVprMgE DNA, while the negative control contained p-fast BAC DNA. Slight banding patterns that match the positive control's presence of the WNVprMgE insert can be seen in clones 1 and 3.

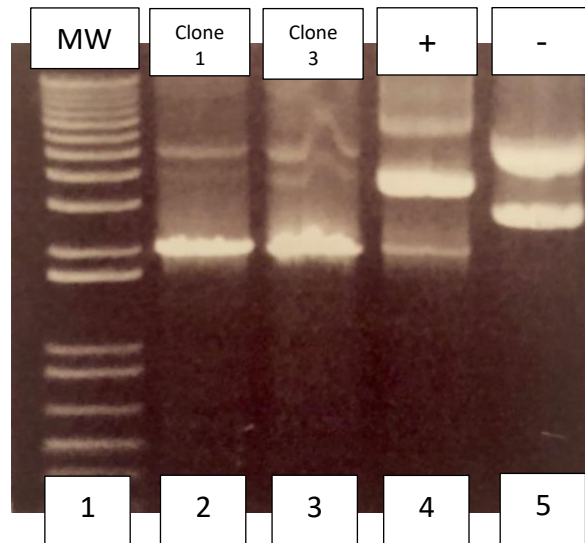


Figure 17 - UV image of PCR reaction of p-fast BAC + WNVprMgE clones 1 & 3 w/ pos. and neg. controls

Electroporation of p-fast BAC into the VZV BAC:

Clone 3 was selected to be electroporated into the VZV BAC + LacZ-attachment site at Tn7 [NEB5- α clone 4.1 (9/20/2017)]. The bacterial artificial chromosome (BAC) of the VZV vaccine contains an attachment (*att*) site at the Lac-Z gene in the Tn7 region, the goal is to successfully attach the p-fastBAC WNVprMgE to the attachment site, thus disrupting the LacZ gene. The clones were plated onto LB plates containing Xgal in order to conduct blue/white screening of the clones to determine if the LacZ gene had been disrupted. Xgal is a substrate which is designed so that the enzyme created by the LacZ gene (beta-galactosidase) will convert the Xgal into a blue color. White clones were selected and picked onto a separate plate, because the absence of the blue coloration confirmed that the LacZ gene was not converting the Xgal into a blue color, which means that the LacZ site had been disrupted, thus inferring that the WNV insert had been successfully cloned into the attachment site.

CONCLUSION:

In this study, it was shown that it is possible to clone the WNVprMgE gene into a VZV BAC. The next step of this study would be to successfully transfect Mewo cells (Human melanoma cells) with the isolated DNA and observe expression of the WNVprMgE antigens through an immunofluorescence assay using primary and secondary antibodies. Unfortunately, due to time constraints we were not able to carry out these next steps, but it is something that will be a focus of Dr. Gray's lab in the future. The important take-away of this study is the affirmation that a rVZV vaccine is potentially a very viable tool for vaccination against WNV and many other diseases which present a molecular antigen complex.

CHAPTER IV: A SUMMARY & CONCLUSION

WNV is a disease which still plagues many parts of the world, and it is clear that a vaccine is necessary. This study attempts to offer a possible solution to different vaccination methods that could prove to be extremely viable options. The process that has been started will hopefully push the development of a viable vaccination method that can be used worldwide. In this study we managed to construct a DNA vaccine and accomplish successful transfection, and construct a recombinant VZV vaccine, both expressing the Premembrane and Glycoprotein E antigens of the West Nile Virus. The transfection of the rVZV vaccine needs additional work, which will be carried out by Dr. Gray and those who work in his lab in the near future, but the methodology shows very promising results.

While the vaccines were created, and we proved that the DNA vaccine could potentially be effective, there is still much more to do in the arena of virology and medical standards for it to be viably useful clinically. This is due to the fact that although successful expression of the proteins in animal cells isolated from specific tissues was demonstrated, we do not know how effective it would be in the human body and the potential side effects that could be induced in a human system. These vaccines would need to undergo extensive animal trials and show promising results to even make it to the human clinical trials stage. Even then, it is known in the scientific world that approximately less than half of the experiments that make it past animal trials show

success in clinical trials. (20) Nevertheless, this study has proven its potential for use, especially since the WNV antigens were successfully cloned into the rVZV vaccine, which opens up several doors of possibility into a viable vaccination method. Personally, this study has provided me with ample valuable experience in the world of microbiological laboratory study and has challenged me more than I imagined it would. I have gained an astronomical amount of respect for those that conduct the research that is so vital to the medicinal world. Until now, I could never grasp the immense amount of work that goes into any and every thing that is used in the medical field. I now truly understand that any advancement, no matter how big or small, is only made possible through the hard work and endless hours devoted by research scientists. I am very grateful for the opportunity to conduct this research and compose this comprehensive thesis.

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